



## Anti-Respiratory Syncytial Viral (RSV) Activity in the Extract of Green Mussel, *Perna viridis* (L)

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**ABSTRACT:** The presence of anti-Respiratory Syncytial Viral (RSV) activity in the extracts of green mussel (*Perna viridis* L) was carried out using human RSV (Sub type-A haem-agglutination titer 1:512). Results of the reducing action of the virus strain A-RSV on fragments of Chorio-allantoic Membrane (CAM) for *in vitro* assay and the Expected Infectious Doses (EID<sub>50</sub>) values obtained against the crude mussel extract, indicated a significant antiviral activity (reduction of infection by 66.66%) when the Haem-agglutination Assay (HA) was performed. However, the aqueous fraction showed a very weak activity (13.63%) as compared to the extract prepared in methanol. The percent inhibition of 86% indicated a significant anti-Respiratory Syncytial Viral activity in the fraction obtained in methanolic extract. The reduction in virus infection in the fraction prepared in methanol showed a very high potential to control the Respiratory Syncytial Viral disease in human.

**Keywords:** Anti-Respiratory Syncytial Viral activity, green mussel, *P. viridis*

### INTRODUCTION

Respiratory Syncytial Virus (RSV) is a major respiratory disease common among infants and young children. However, the group also includes premature infants, older adults and people of any age who are at high risk of severe disease cause by RSV or even death (Figueras-Aloy *et al.*, 2004). The virus causes acute headache, cough, fever and weakness to the patient. Infection cause by Respiratory Syncytial Virus in premature babies and kids leads to even more complications affecting severely the lungs, heart and immune system. RSV infection primarily manifests as bronchiolitis or pneumonia resulting in ~75,000 to 125,000 hospitalizations in the United States, every year (Leader and Kohlhase, 2003; Brief Report, 2007)). The virus is highly contagious and can be spread through droplets when an infected person coughs or sneezes (Hall and Douglas, 1981). The virus can also survive on surfaces such as countertops or doorknobs and on hands and clothing which can easily be spread when a normal person touches an object or surface contaminated with the virus. It has been reported that

almost all young children are infected with RSV at least once in their life time.

Unfortunately, so far, no effective preventive or therapeutic drug has been developed to control RSV infection. The modern antibiotics are not used because RSV is a virus and antibiotics are only effective against bacteria. The patients are normally treated for controlling fever using a non-aspirin medicine like acetaminophen etc. Aspirin is not recommended to control fever in children infected with virus as this drug results in Reye syndrome - a life-threatening illness.

Recent studies showed that green mussels are not only an inexpensive source of protein for human consumption but also possess some complex bioactive compounds that have potential in bio-medical science. A team of Russian scientists, in 1969, discovered an antiviral compound in the meat of blue mussel and opened a new chapter in biomedical science. An extract prepared from the brown mussel (*Mytilus edulis*), a sister species of green mussel, has been found to have both prophylactic and therapeutic properties. It can cure viral diseases such as influenza, *Herpes simplex*, *Herpes zooster*, hepatitis and flu.

The use of mussel hydrolyzate among children at kindergarten and pre-school centers has been found to reduce incidence of infectious diseases and post infection effects by four times. Among children, use of mussel hydrolyzate for two months has reduced the incidence of flu by five times lower than other children who did not use it.

Considering the importance of the mussel extract in protecting and curing viral infections, an attempt has been made to reexamine the presence of an anti-Respiratory Syncytial Viral activity in the extract of green mussel, *Perna viridis* (L).

## MATERIALS AND METHODS

In the present investigation, anti-Respiratory Syncytial Viral studies were conducted on human RSV (Sub type-A haemagglutination titre 1:512) with the help of Pasteur Institute of Epidemiology and Microbiology, St. Petersburg, Russia. The following protocol was followed to assess the anti-viral activity in the mussel extract:

Samples of live green mussel (*P. viridis*) measuring in shell length 30-40 mm were collected directly from the rocky shores. The mussels were cleaned and deshelled with the help of a sharp knife and meat and mantle fluid was removed carefully in the laboratory. The meat with mantle fluid was transferred in a double necked round bottom distillation flask to which protosubtiline enzyme prepared from the *Bacillus subtilis*, was added at the ratio 6% of the weight of the meat with 6% distilled water. The distillation flask was placed on a heating mantle for fermentation at a constant temperature of 40°C for two hours. The resultant solution in the form of thick paste was digested with concentrated hydrochloric acid (12% of the total meat weight) for 22 hours at 100 + 2° C. After the completion of the digestion and distillation process, the resulting solution was allowed to cool to room temperature. The solution was neutralized with sodium hydroxide to achieve 5.6 pH.

The active extract was isolated from the rest of the solution by keeping it in a separating flask for 10 days and carefully removing the middle part of the solution after the formation of different layers. The crude extract was cooled in a deep freezer to -80°C and lyophilized at -20° C (~10 hours). The lyophilized material was dissolved in methanol with stirring using a magnetic stirrer (3-4 hours). The solution was then allowed to settle and filtered using ordinary filter paper to yield clear methanol solution and a residue. The process was repeated twice to separate all the methanolic soluble part, completely. The residue was dissolved in double distilled water.

## Assessment of antiviral activity in the mussel extract

### A. Preparation of extract

The crude, aqueous and methanolic fractions of the extract from green mussel (0.5 ml) was transferred to one test tube containing 4.5 ml of peptonic solution to achieve a dilution of extract to 1/10 times. This was considered as a stock working extract for all experimental purpose.

### B. Preparation of peptonic medium

Gelatin (2.0 g) was thoroughly mixed in 100 ml double distilled water. This was followed by addition of NaCl (8.0 g), KCl (0.6 g), CaCl<sub>2</sub> (0.8 g), MgCl<sub>2</sub> (0.15 g), glucose (0.9 g) and 25 ml of phenol (0.01%). The volume of the solution was made up to 1000 ml with double distilled water. 0.1 g of antibiotic (Penicilline: 1000 units /ml) was also added and pH of the solution maintained between 6.5 and 7.0. The solution was sterilized in an autoclave for one hour at 15 lbs. The pH of the medium was adjusted with NaHCO<sub>3</sub> to achieve a pH of 6.0 before the experiment.

### C. Preparation of virus titer

Stock solutions of RSV (Sub type-A haemagglutination titre 1:512) was diluted in six test tubes with peptonic medium in descending order to achieve virus concentrations from 10<sup>-1</sup> to 10<sup>-6</sup>.

### D. Preparation of *in vitro* experiment with chorioallantoic membrane (CAM) of chick embryo with viral strain- RSV

Three sets of multiwell plates were thoroughly cleaned with soap and washed several times with double distilled water and then with alcohol for sterilization. Two fertilized eggs of chicken (13 days old) were cleaned with freshwater to remove all impurities. The eggs were also cleaned thoroughly with absolute alcohol. A small cut was made at the narrow end of the eggs with a help of a pair of sharp scissors and shell piece was removed. The egg with growing embryo was emptied in a glass bowl. Small pieces of the shell with chorioallantois membrane (5 × 5 mm in size) were cut and transferred immediately in the peptonic medium.

The multiwell plates were divided into three columns with four wells in each column and 6 wells in each row for each sample (crude, methanolic and aqueous mussel extracts). In all well of the multiwell plate, 0.5 ml of peptonic medium was transferred with a sterilized pipette. A single piece of the shell with CAM cut from the fertilized egg was transferred in the wells of multiwell plate so as to completely dip in the peptonic medium. 0.1 ml of mussel extract (crude, methanolic and aqueous) was taken out with the help of a pipette and transferred in all wells column 1 of each set of multiwell plate.

The second column of the plate was identified as control preparation column where only extract of green mussel (1/10) was transferred. The multiwell plate was finally closed properly and kept at a constant temperature of  $34 \pm 1^\circ \text{C}$  for 2 hours.

The multiwell plate was removed from the oven after 2 hours of incubation and in each well of columns 1 and 3 of multiwell plate, 0.1 ml of viral strain was added with descending dilutions i.e. from 1<sup>st</sup> row ( $10^{-1}$ ) to 6<sup>th</sup> row ( $10^{-6}$ ). The virus was not added in the wells of control preparation i.e. column-2. The plate was again closed properly and kept at a constant temperature of  $36 \pm 1^\circ \text{C}$  for incubation for 48 hours. The multiwell plate was removed from the oven after 48 hours and shell pieces taken out carefully with the help of fine tweezers avoiding the mixing of the solution of the wells. In all the three columns of each well of the plate, 0.05 ml of chicken erythrocyte (5%) was added with the help of a pipette. The plate was kept undisturbed at room temperature for 20-30 minutes and then assessed for the presence of antiviral activity by hem-agglutination reaction. Titre of virus causing 50% infection in developing chick embryo expressed as  $\text{EID}_{50}$  (Effective Infectious Dose). The reduction in virus infection was calculated by the following equation:

Percent inhibition =  $(C-T/C)/100$  (Premnathan *et al.*, 1992)

where C and T represent the control and treated with green mussel extract, respectively. The aforesaid process required absolute precaution for pyrogen contamination at all processing steps. All apparatus and reagents used in the present study were therefore, pyrogen free.

## RESULTS

Results of reducing the action of virus strain A-RSV on fragments of CAM for *in vitro* study are presented in Tables 1 and 2. The  $\text{EID}_{50}$  values obtained against the crude mussel extract indicated an antiviral activity (reduction of infection by 66.66%) when the Hemagglutination Assay (HA) was performed (Table 1). However, the aqueous fraction showed a very weak activity (13.63%). This shows that the aqueous fraction of the mussel extract is not suitable for inhibiting the activity of virus strain A-RSV. The reduction in virus infection or percent inhibition 86% indicated a significant anti-Respiratory Syncytial Viral activity in the methanolic fraction of the mussel extract. This shows that fractionation done in methanol has a high potential to control Respiratory Syncytial Viral infection (Table 2).

**Table 1: Results of reducing the viral strain sub type A (RSV) activity on fragments of CAM of chicken embryo with the crude mussel extract.**

Sample	Virus dilution (A/RSV)						$\text{EID}_{50}$ (Ig)	Percent inhibition
	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$		
Control virus	++ ++	++ ++	++ ++	++ ++	++ ++	+ - + -	6.00*	-
Crude extract of <i>P. viridis</i>	++ ++	+ - + -	- - - -	- - - -	- - - -	- - - -	2.00	66.66

[Positive (+) shows the presence of virus, Negative (-) shows the absence of virus and \*0.5 a constant added to each value]

**Table 2: Results of reducing the viral strain sub type A (RSV) activity on fragments of CAM of chicken embryo with aqueous and methanolic fractions of mussel extract.**

Sample	Virus dilution (A/RSV)						$\text{EID}_{50}$ (Ig)	Percent inhibition
	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$		
Control virus	++ ++	++ ++	++ ++	++ ++	++ ++	- - - -	5.50*	-
Aqueous extract of <i>P. viridis</i>	++ ++	++ ++	++ ++	++ ++	+ - - -	- - - -	4.75	13.63
Methanolic extract of <i>P. viridis</i>	+ - - -	- - - -	- - - -	- - - -	- - - -	- - - -	0.75	86.63

[Positive (+) shows the presence of virus, Negative (-) shows the absence of virus and \*0.5 a constant added to each value]

## DISCUSSION

The extract prepared by enzyme–acid hydrolyzing process using meat and mantle fluid of the green mussel (*P. viridis*) and other commercially important marine bivalves indicated the presence of high antiviral activity in both *in vitro* and *in vivo* assays (Chatterji *et al.*, 2002). The extracts of black clam (*V. cyprinoids*) and mud clam (*P. erosa*) have been reported to reduce the infection of influenza virus type-A considerably, when the tests were carried out on chicken embryo *in vivo* (Chatterji *et al.*, 2002). Similar observations were made using the extract prepared from the Russian blue mussels, where influenza virus types A and B were found to reduce infectious activity by 3.0–6.0 lg EID<sub>50</sub> (Bichurina *et al.*, 1994). The treatment of toxigenic influenza-infected mice with the extract prepared from the blue mussel showed 66–84% survival (Bichurina *et al.*, 1994). Administration of mussel extract showed a significant protective effect from various viral infections in mice. Maximum prophylactic effect has been observed in mice when a dose of mussel extract (both through intranasal and oral) was given 5 h before inoculation of virus and the mice showed 100% survival ((Bichurina *et al.*, 1994).

The extract prepared from brown mussel commonly known as mussel hydrolyzate with a trade name MIDEL in Russia. The MIDEL showed high immunomodulation properties. It has already been approved by the Drug and Food Controller in Russia after all clinical, toxicological and pharmacological tests (Boikov *et al.*, 1997). The drug is now available in the Russian market for common use. It is surprising to know that the drug has been proved to be more effective in people living under conditions of high background of nuclear radiation, unfavorable ecological condition and thickly populated areas such as factories, schools, institutes and army divisions. As the viral infection spreads rapidly through schools, child-care centers and thickly populated areas, the Russian Government has strongly recommended the use of mussel hydrolyzate (MIDEL) on a regular basis.

RSV infection often occurs as an epidemics causing bronchiolitis or pneumonia that usually lasts about a week. However, in some cases it may take several weeks for recovery (Welliver, 1998; Malhotra and Krilov, 2000). RSV is typically identified in nasal secretions, which can be collected either with a cotton swab or by suction through a bulb syringe. The prevention of serious RSV-related respiratory disease among young children, a monthly dose of an injection consisting of RSV antibodies is being recommended during peak RSV season from November to April (McCarthy and Hall, 1998).

As the effect of this injection is for a short duration, it has been recommended to be administered in subsequent years until the child is no longer at the high risk for severe RSV infection.

In our investigation crude extract prepared from the green mussel by the enzyme hydrolysis process showed relatively a less activity on the reduction of virus. However, the activity increased considerably in one of the fractions (fraction prepared in methanol) when an attempt was made to semi purify the mussel extract. Based on our findings conducted on RSV, it is expected that the methanolic extract prepared from the green mussel would be useful in developing an effective prophylactic and therapeutic anti-viral drug. The same drug may also be active on similar viruses including severe acute respiratory syndrome (SARS) that is common in Hong Kong.

## CONCLUSIONS

The present study showed that the green mussel has a great potential in developing useful anti-viral drug specially to control respiratory syncytial viral infections among children and old aged people. The EID<sub>50</sub> values obtained against the crude mussel extract indicated an antiviral activity (reduction of infection by 66.66%) when the Hemagglutination Assay (HA) was performed. The aqueous fraction showed a very weak activity (13.63%) as compared to the methanolic extract where percent inhibition 86% indicated a significant anti-Respiratory Syncytial Viral activity. It is expected that the methanolic extract prepared from the green mussel would be useful in developing an effective prophylactic and therapeutic anti-viral drug. The isolation and purification of active component from the green mussel extract will certainly be helpful in protecting and treating various viral diseases in human beings, in near future.

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